Vitellogenin Gene Expression in Fathead Minnows Exposed to EE2 in a Whole Lake Dosing Experiment

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Introduction

There is increasing concern about the potential impact of endocrine disrupting compounds (EDCs) on aquatic organisms. EDCs are compounds that interfere with the normal functioning of hormones in the body. Among the EDCs that are found in aquatic habitats are synthetic estrogens, which are used in contraceptives and other pharmaceuticals. These chemicals enter waterways through sewage treatment plants, and are also found in surface waters (Lange et al 2001). One of the most commonly used synthetic estrogens is 17-α-ethynyl estradiol.

Vitellogenin is an egg yolk protein precursor that is produced by female fish prior to spawning. Its synthesis is initiated in response to rising circulating estrogen levels. Males do not normally produce this protein, but exposure to estrogenic contaminants initiates its production. Plasma vitellogenin levels in males exposed to estrogenic substances can rise several thousand fold, to levels as high as or higher than that found in females (Palace et al. 2002).

Although the presence of plasma vitellogenin protein can be used as an indicator of exposure to estrogenic compounds, our laboratory has developed a reverse transcription-PCR method for quantifying vitellogenin gene messenger RNA (Lattier 2002). Because the presence of circulating protein occurs much later than transcription of the vitellogenin gene and is modulated by numerous control mechanisms, quantitation of vitellogenin gene transcription is potentially a more sensitive and immediate indicator of exposure.

In order to investigate the effects of an endocrine disrupting compound on a whole lake ecosystem, Fisheries and Oceans Canada dosed a lake in the Experimental Lakes Area (ELA) of northwestern Ontario with 17-α-ethynyl estradiol (EE2) from late May to October 2001 (Figure 1). The U.S. EPA collaborated in this study by evaluating vitellogenin gene expression in fathead minnow.

The goals of this study were to compare vitellogenin gene expression in 1) indigenous male fathead minnows exposed over a period of weeks or months; 2) fish moved from a reference lake into the dosed lake for a period of days; 3) minnows exposed for 48 hours to water samples sent to Cincinnati, OH; and 4) fry exposed for five days to sediment from the dosed lake.

Materials and Methods

Study Site

Both the study lake and the reference lake are small, Precambrian Shield lakes located in the Experimental Lakes Area (ELA) in northwestern Ontario, Canada. The study lake (Lake 260) has an area of 34 ha. It was dosed with EE2 from late May to October 2001. Lake 114, with an area of 12 ha, was used as a reference lake. Details regarding the dosing of this lake can be found in Palace et al. (2002).

Figure 1. View of Lake 260 in the Experimental Lakes Area.

Animals

Fathead minnows, *Pimephales promelas*, were used for all experiments. As described below, most of the fish used were from either the reference or study lake. In addition, sexually mature male fathead minnows and 24 to 48-h-old fry, raised in the Cincinnati, Ohio U.S. EPA aquaculture facility, were used. For all analyses, replicates of five fish were used.

Materials and Methods

Exposure Protocols

Indigenous Fish: Indigenous fish were collected from Lake 260 after six weeks, eight weeks and sixteen weeks of dosing. Minnows were collected from Lake 114 at the same timepoints. Fish were trapped in standard minnow traps. Males were identified by external morphology. One set of females collected on 25 July was used for analysis. All other females were returned to the lake of origin.

Deployment study: Fish were collected from Lake 114. Male fish were deployed in both Lake 114 and 260. Fish were housed in net enclosures that were suspended 1-2 m below the surface. Fish were not provided with supplementary food. After 1, 3, 7 and 13 days fish were removed for analysis. Five fish were removed from an enclosure in each lake at most timepoints.

Exposure to sediment elutriate: Sediment samples (1-2 liters) were collected from five locations in Lake 260 and a single location in Lake 114. Sediment was shipped on ice to Cincinnati. Sediment was combined with two volumes of water, shaken for one hour, and centrifuged. Liquid phase (elutriate) was used for exposures. Due to the limiting volumes of water available, fry were used for exposures instead of adults. Fry were exposed to elutriate for five days, with elutriate renewed daily

Exposure to grab Samples: Water was collected from Lakes 114 and 260. Samples were shipped on ice to Cincinnati. Male fathead minnows raised in the Cincinnati aquaculture facility were exposed to water samples for 48 hours with water renewal after 24 hours. Additional male minnows were exposed to 5 ng/L ethynylestradiol for the same period of time.

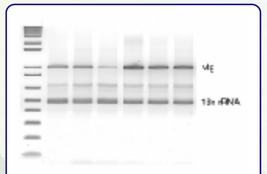

Figure 2. Agarose gel showing vitellogenin and 18S ribosomal bands

RNA Preparation and RT-PCR

Protocols for RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR can be found in Lattier et al. (2002). Briefly,

1. Adult liver tissue or pooled fry was suspended in RNA*later* (Ambion Inc., Austin, TX).

2. Total RNA was isolated using the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987).

3. The integrity of the RNA was determined by visual inspection of the 18s and 28s ribosomal bands on a formaldehyde/MOPS gel.

4. Reverse transcription was performed using GeneAmp® RNA PCR reagents (PE Applied Biosystems, Foster City, CA). PCR reactions used Advantage-2 DNA polymerase (CLONTECH Laboratories, Palo Alto, CA, USA.) with gene-specific oligonucleotides and an empirically determined volume of 18S Competimer®/universal ribosomal RNA (rRNA) primer mix (Ambion Inc., Austin, TX) in a muliplex reaction.

5. PCR products were separated on 1.8% agarose gels (Fig. 2). Gels were stained with SYBR® Green I (Molecular Probes, Eugene, OR) and scanned using a FluroImager® 595 system (Molecular Dynamics, Sunnyvale, CA). Relative intensities for both the Vg and 18S ribosomal bands were analyzed with ImageQuant® (Molecular Dynamics) software.

6. The pixel density ratios of relative gene expression [Vg/(Vg+18S)] were averaged and standard deviations calculations using routines available in Lotus 123 and Microsoft Excel.

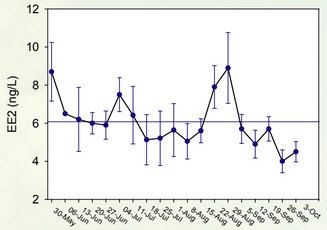

Figure 3. Concentrations of EE2 in epilimnion of Lake 260. Adapted from Palace et al. (2002)

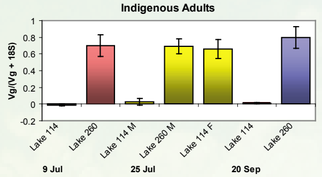

Figure 4. Vitellogenin gene transcription in male fathead minnows after long-term exposure to EE2

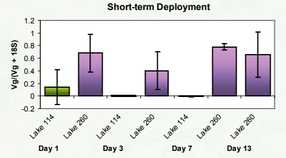

Figure 5. Vitellogenin gene transcription in male fathead minnows after short-term deployment in lake dosed with EE2.

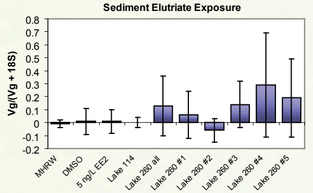

Figure 6. Vitellogenin gene transcription in fry after five-day exposure to elutriate from sediment collected from lake 114 and five sites from lake 260.

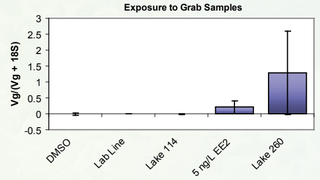

Figure 7. Vitellogenin gene transcription of male fathead minnows exposed for 48 hr to water collected from the lake dosed with EE2.

Results and Discussion

EE2 concentrations in Lake 260

Concentration of 17-α-ethynyl estradiol in the epilimnion of Lake 260 from May to October 2001 ranged from around 4 to 8 ng/L (Fig. 3). The mean concentration (± SD) was 6.0 ± 2.8 ng/L. Concentrations were maintained very near the target concentration, and are environmentally relevant. Estrogens have been found in effluents from sewage treatment plants at concentrations in this range (Larsson et al 1999).

Long-term exposure

Vitellogenin gene transcripts were nearly undetected in male fish from the control lake, Lake 114 (Fig. 4). Male fish sampled from the dosed lake, Lake 260, on 9 July (after six weeks of dosing) showed high levels of vitellogenin gene expression. These levels were maintained throughout the dosing period. Vitellogenin gene expression was comparable to that seen in females sampled on July 25.

Short-term deployment

After only a single day of exposure, male fathead minnows exhibited high levels of vitellogenin gene transcription (Fig. 5). Levels were equivalent to those seen in indigenous fish exposed for six weeks. Again, the levels remained high throughout the 13-day exposure. Male fish from the Lake 114 showed almost no vitellogenin expression. A single fish from this lake had elevated vitellogenin levels. This could be due to the misidentification of a female fish as a male, but there is no way to verify this. Due to mortality, only four fish were available for analysis from the Lake 260 enclosure on day 13, and none remained in Lake 114 on day 13.

Sediment elutriate exposures

Highly variable gene expression was found in fry exposed to dosed lake sediments (Fig. 6). There was no significant gene expression in fry exposed to control lake sediments.

Grab Samples

Fish exposed to Lake 260 water sent to the U.S. EPA facility in Cincinnati, OH showed elevated vitellogenin gene expression, although there was much variation (Fig 7). Two of the five fish had highly elevated vitellogenin levels, while one exhibited no increase.

Plasma vitellogenin protein levels in male fathead minnows from Lake 260 increased substantially during the dosing period (Palace 2002). Concentrations in these fish were 9000-fold higher than that from males collected from reference lakes or fish collected before dosing.
Histological analyses of male fathead minnows from the dosed lake showed alterations in tissues of the gonad, liver and kidney and Palace et al. (2002). Histopathic changes included fibrosis of testes and inhibited development of testicular tissue. Enlargement of hepatocytes within the liver, and thickening of Bowman's capsules and necrosis of tubules in the kidney were among the changes found. Effects in these tissues were likely due to the production of vitellogenin in the liver and accumulation in the kidney.

Detection of increased plasma vitellogenin levels is a reliable indicator of exposure to estrogenic compounds. However, it may take two to three weeks for circulating plasma vitellogenin levels to increase to significant levels (Schmid et al. 2001). We have shown that increases in vitellogenin gene transcription occur after only a single day of exposure to environmentally relevant levels of EE2. The use of this method as an exposure indicator for the presence of estrogenic compounds in aquatic environments, given that it is so rapid, has great potential.

Conclusions

- Male fathead minnows exposed to EE2 at concentrations of around 6 ng/L exhibit significant upregulation in vitellogenin gene transcripts. This level of transcription does not diminish after a long-term exposure (nearly four months).
- Fathead minnows exposed to EE2 at these concentrations for as little as 24 hours exhibit similar upregulation in vitellogenin gene transcription.
- Vitellogenin gene transcription is a promising tool as an indicator of exposure to environmental estrogens.

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